Preparative isolation of apple flavan-3-ols monomers and oligomers using pH-zone-refining centrifugal partition chromatography combined with reversed-phase liquid chromatography

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Preparative isolation of apple Flavan-3-ols monomers and oligomers using pH-zone-refining centrifugal partition chromatography combined with preparative reversed-phase liquid chromatography

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Abstract

Flavan-3-ols (catechin monomers and procyanidins) are the main class of polyphenols in apples and are found in high concentrations in cider apple varieties. They are known to be involved in bitterness and astringency in apple-based beverages, and also contribute to polyphenol nutritional intake.

Therefore, highly purified flavan-3-ol fractions isolated from raw materials are needed to study their various properties. For this purpose, a gentle strategy combining pH-zone-refining centrifugal partition chromatography (pH-ZRCPC) and preparative reversed-phase liquid chromatography (Prep-RPLC) was developed to recover one hundred milligrams of a high purity apple flavan-3-ol fraction.

First, pH-ZRCPC fractionation in descending mode was optimized to remove hydroxycinnamic acid derivatives using a biphasic mixture composed of ethyl acetate/*n*-butanol/water (3/2/5, v/v). Trifluoroacetic acid and sodium hydroxide were used as retainer and eluter, in the upper and lower phases, respectively. Secondly, Prep-RPLC separation was carried out in isocratic mode at 20% ACN to remove dihydrochalcones. Finally, from one gram of a crude polyphenol extract, four hundred and nine milligrams of a highly purified

fraction of flavan-3-ols with an average degree of polymerization close to 3.1 was obtained with 73% recovery.

Keywords: pH-zone-refining CPC, Tannins, Procyanidins, Hydroxycinnamic acids derivatives, Reversed-Phase preparative HPLC

1 Introduction

Polyphenols are a huge family of complex secondary metabolites exclusively synthesized in the plant kingdom. Based on their chemical structure, they are subdivided into several classes including flavonoids. These ones are the most common class of polyphenols in the human diet. The main dietary source of flavonoids is fruit (in particular berries and citrus fruits) and vegetables, but a large amount can also be found in dark chocolate, extra-virgin olive oil, and some beverages (tea, coffee, wine, and apple cider) [1,2]. In recent decades, numerous studies have established their health benefits. In particular, flavan-3-ols, are the most structurally complex subclass of flavonoids which have been recognized as antihypertensive agents [3]. They have been also reported to have multiple biological effects, mainly attributed to their antioxidant properties, as they can act as chain breakers or radical scavengers [4]. Moreover, mounting evidence indicates that a higher intake of flavan-3-ols rich foods is closely linked to a reduction in chronic-degenerative diseases such as type 2 diabetes, cardiovascular diseases, and some types of cancer [5,6]. Moreover, procyanidins (i.e. flavan-3-ol oligomers and polymers or condensed tannins), can interact with other macromolecules such as polysaccharides or proteins [7]. This property is responsible for the perception of astringency resulting from interactions of tannins with salivary proteins [8], the formation of haze and precipitates in beverages [9], as well as the inhibition of enzymes and the reduced digestibility of macromolecules [10].

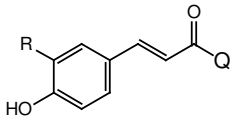
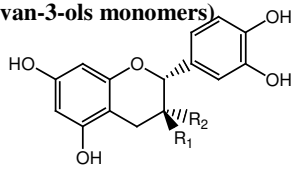
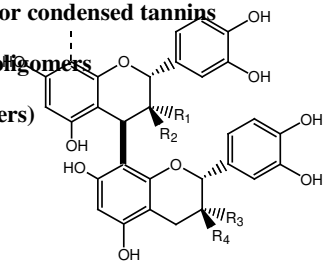
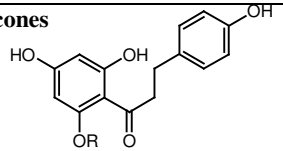
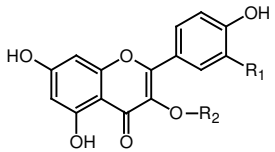
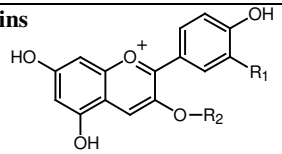
Cider apples are naturally rich in several classes of polyphenols including flavan-3-ols (FA), hydroxycinnamic acid derivatives (HCA), flavonols (FO), and dihydrochalcones (DHC), whereas anthocyanins (AN) are generally found in lower concentration. The different structures are presented in Table 1. 5'-O-caffeoylquinic acid (CQA) is the most common HCA, with average amounts close to 640 mg/kg of fresh matter (FM) [11]. The main DHC are phloridzin and phloretin xyloglucoside with average amounts close to 40 and 30 FM mg/kg, respectively. FO and AN are mainly located in peel and are present in low concentrations in apple juices and ciders [12,13]. In cider apples, flavan-3-ols are divided into two subclasses. The monomeric forms, namely the catechins, include mainly (-)-epicatechin

with average contents of 350 mg/kg FM, and (+)-catechin in much lower concentrations around 50 mg/kg FM. The second flavan-3-ol subclass includes catechin oligomers and polymers, namely procyanidins or condensed tannins. They are comprised mainly of (-)-epicatechin units and are the most abundant polyphenols in both table and cider apples with an average concentration ranging from half a gram to several grams per kg FM, depending on the varieties. Their average degree of polymerization (aDP) is generally between 4 and 7, except for some cider apple varieties that exhibit aDP up to 50 [11].

The need to recover pure and native tannin fractions from various raw materials or apple-derived products is essential to explore their numerous properties such as tanning properties, nutritional effects, and biological activity. However, this task is particularly difficult due to their polydisperse nature and the balance between their hydrophilic and hydrophobic characters that finally vary little between classes. For instance, reversed-phase HPLC with a classical gradient using a water/organic solvent mixture elutes apple procyanidins throughout the chromatogram without any clear separation from HCA, FO, or DHC phenolic classes [14]. Only procyanidin oligomers could be observed as well-resolved peaks.

Many studies have been conducted to purify and fractionate condensed tannins. Gel filtration chromatography with Sephadex® LH-20 or Toyopearl HW40F has been used to fractionate procyanidins and phenolic acids directly from cider [15] or apple [16]. Several studies have succeeded in isolating procyanidin oligomers from different plant sources using this technique [17,18]. However, all these conventional methods are tedious, time-consuming, and solvent-wasting, with the risk of losing some of the tannins through irreversible adsorption on the solid chromatographic phases. Others methods have been developed to selectively adsorb the tannin fractions on miscellaneous materials such as polyvinylpolypyrrolidone [19–21] or deacetylated glucomannan [21]. However, the desorption of tannins is either difficult or too drastic leading to their degradation. Indeed, desorption of tannins from deacetylated glucomannan is possible by increasing the pH but leads to their partial degradation through autoxidation [22].

89 Table 1. List of different structures of phenolic compounds found in apple.

Phenolic classes	Compounds	Average contents (mg.kg^{-1} FM) [Min; Max]	References
Hydroxycinnamic acid derivatives 	R = H: 4-O-*para*-coumaroylquinic acid R = OH: 5'-O-caffeoylquinic acid Q: quinic acid	90 mg.kg^{-1} FM [30-180] 640 mg.kg^{-1} FM [150-1200]	[13,23]
Catechins (flavan-3-ols monomers) 	R_1 = OH; R_2 = OH: (+)-catéchin (CAT) R_1 = H; R_2 = OH: (-)-epicatéchin (EC)	50 mg.kg^{-1} FM [t-150] 350 mg.kg^{-1} FM [t-1400]	[11,23,24]
Procyanidins or condensed tannins (flavan-3-ols oligomers and polymers) 	DP2: Dimers (B1, B2, B5) DP3: Trimers (C1) DP4: Tétramers (D1)	Procyanidin B2: 280 mg.kg^{-1} FM [20-590] PCA (B2 included): 2030 mg.kg^{-1} FM [500-3280]	[2,23,25]
Dihydrochalcones 	R = Glu: phloridzin R = Xyl-Glu: phloretin xyloglucoside	40 mg.kg^{-1} FM [20-100] 30 mg.kg^{-1} FM [10-100]	[13,24]
Flavonols 	R_1 = OH; R_2 = Glu: isoquercetin R_1 = OH; R_2 = Gal: hyperoside R_1 = OH; R_2 = Rham: quercitrin R_1 = OH; R_2 = Xyl: reynoutrin	Total Flavonols: 55 mg.kg^{-1} FM [t-80]	[12,13]
Anthocyanins 	R_1 = OH; R2 = Gal: ideain ou Glu ou Ara ou Xyl	Total Anthocyanins: 10 mg.kg^{-1} FM [t-35]	[13,26]

90 FM: fresh matter; t: traces; Ara: arabinose; Gal: galactose; Glu: glucose; Rham: rhamnose; Xyl: xylose; PCA: procyanidins.

Interestingly, Centrifugal Partition Chromatography (CPC) is an alternative tool to isolate tannins from complex mixtures. This method, which does not need a solid adsorbing support for the fractionation, can be performed by adapting the two-phase solvent system to separate target components according to their partition coefficients [27]. Few studies have been devoted to the separation of polyphenols using this technique [28–32]. Esatbeyoglu *et al.* [28] successfully purified procyanidin dimers from cocoa bean extract using CPC. However, in this particular case, the crude extract used for the fractionation contained neither hydroxycinnamic acid derivatives nor highly polymerized procyanidins [28]. Furthermore, Kohler *et al.* managed to separate oligomeric tannins (dimers to tetramers) from grape seeds after removal of polymeric procyanidins using solvent precipitation [30]. Last, several studies deal with the fractionation of apple procyanidins using CPC, but the separation was conducted on an initial extract containing only flavanols without other phenolic classes [33–35].

pH-ZRCPC is a recent CPC separation technique developed by Ito *et al.* as a preparative purification method for the separation of compounds whose electric charge is pH-dependent [36]. In addition to the conventional CPC principle, this technique is based on the fractionation of ionisable compounds (acids or bases). For the fractionation of acidic organic compounds, an acid (retainer) and a base (eluter) are added in low concentrations to the stationary and mobile phases, respectively. Initially, the crude extract is acidified with the retainer whose p*K*a has to be lower than that of the analytes to be fractionated. This acidification converts the analytes into their protonated forms, which are preferentially partitioned in the stationary apolar phase. Following injection, the elution with the aqueous mobile phase containing the basic eluter triggers a deprotonation process. Consequently, organic acids become less hydrophobic and are better partitioned in the mobile phase. The fractionation principle is properly described by Ito [37]: the retainer in the stationary phase is gradually neutralized by the eluter in the mobile phase, forming a sharp retainer border that travels along the column at a constant rate, substantially lower than that of the mobile phase. The analytes are eluted after the retainer border, according to their p*K*a and hydrophobicity, as fused rectangular peaks. Compared with conventional CPC, pH-ZRCPC has many advantages such as a higher sample loading capacity, minimum overlap of rectangular peaks, and concentration of analytes initially present in low amounts (considered as contaminants) as sharp peaks at the rectangular peak boundaries [37,38]. This technique has been used to separate various kinds of natural products such as fluorescein-type compounds [39,40], basic compounds [41], peptides [42], polar alkaloids [43–47], highly-polar sulfonic acids [48], and

organic acids [38,49–51]. Studies have been carried out to isolate caffeoylquinic acid isomers [52] or to separate CQA from caffeic acid [53] using pH-ZRCPC, but the solvent systems and parameters were only optimized to fractionate HCA without taking into account the recovery of tannins.

The aim of this work was to obtained a highly purified flavan-3-ol fraction containing catechin monomers and procyanidin oligomers but devoid from others phenolic components. For this purpose, a gentle strategy combining pH-zone-refining centrifugal partition chromatography (pH-ZRCPC) and preparative reversed-phase liquid chromatography (Prep-RPLC) was developed. First, pH-zone-refining CPC (pH-ZRCPC) was used for the primary fractionation to remove HCA from a crude apple polyphenolic extract, as hydroxycinnamic acid derivatives are more ionisable than other phenolic compounds due to their carboxylic function. Secondly, preparative reversed-phase liquid chromatography (Prep-RPLC) was used to recover the high purity flavan-3-ol fraction.

2 Material and methods

2.1 Reagents

Acetonitrile, ethyl acetate, butan-1-ol, and methanol were purchased from Carlo Erba Reagents (Val de Reuil, France). Formic Acid was provided by Sigma-Aldrich (St. Louis, MO). Sodium hydroxide 50% was purchased from VWR BDH Prolabo (Fontenay-sous-Bois, France), and Trifluoroacetic acid from Thermo Fisher (Kandel, Germany). Ultrapure Water was purified using the Elga Purelab system (Labelians, Nemours, France). The others reagents used for preparation of the crude apple polyphenolic extract and phloroglucinolysis are listed in Millet *et al*. [54] and Le Deun *et al.* [55], respectively. (+)-catechin (CAT), (-)-epicatechin (EC), 5-O -caffeoylquinic acid (CQA), 4-O-para-coumaroylquinic acid (PCQ), and phloridzin (PLZ) standards were provided from Sigma-Aldrich (St. Louis, MO). Hyperoside (QCE-3-O-GAL), procyanidin dimer B1 (PA_B1) and procyanidin dimer B2 (PA_B2) standards were obtained from Extrasynthese (Genay, France).

2.2 Preparation of the crude apple polyphenol extract

Phenolic compounds were extracted from a French cider apple cultivar, named ‘Marie Menard’, which is known for its high polyphenol content, close to 32 g/kg dry matter (DM), and in particular procyanidins (close to 20 g/kg DM with aDP 4.6) [56]. Extraction was carried out according to the method described by Millet *et al.* [54]. Briefly, non-oxidized apple juice was produced from ‘Marie Menard’ cider apples collected from our experimental

orchard (IFPC, France). The clarified juice was loaded on an Amberlite FPX66 resin (Rohm and Haas Company, Philadelphia, USA) primarily to remove sugars, organic acids, and salts, and to retain polyphenols. After rinsing the resin with acidified water (1‰ v/v acetic acid), the polyphenolic fraction was subsequently eluted using 96% ethanol. The solvent was removed using rotary evaporation and the concentrated polyphenol fraction was freeze-dried.

The detailed polyphenol composition of this crude extract of total native polyphenols, named "TotPP", was characterized using UPLC-UV/Visible-MS (see section 2.5). The method adapted from Guyot *et al.* enabled the concentrations of the phenolic compounds to be measured including hydroxycinnamic acids derivatives, catechins (monomers), low molecular weight procyanidins, dihydrochalcones, and flavonols [14]. The total flavan-3-ol concentration (including catechin monomers and procyanidins) and the average polymerization degree (aDP) were determined using UPLC-UV/Visible-MS analysis of the procyanidin cleavage products after phloroglucinolysis [57], as described by Le Deun *et al.* [55]. Phloroglucinolysis is a depolymerization technique that allows to specifically quantify terminal and extension units of procyanidins present in samples and fractions. The aDP of flavan-3-ols corresponds to the molar ratio of total units on terminal units.

2.3 pH-zone-refining centrifugal partition chromatography (pH-ZRCPC)

2.3.1 Determination of partition coefficients in solvent systems

A preliminary experiment was conducted using four biphasic solvent systems adapted from Oka [58] to determine the partition coefficient of the target compounds for a conventional CPC (without the addition of an acid or base in the solvent system). The solvent systems used were ethyl acetate:*n*-butanol:water with 5/0/5, 3/2/5, 2/3/5, and 1/4/5 (v/v) named systems A, B, C and D, respectively.

The TotPP extract was dissolved at 2 g/L in a mixture of methanol/water (50/50). One mL of solution was distributed in 10-mL glass tubes and evaporated under vacuum using a Genevac (Model EZ-2). Two mL of upper and lower phases were added to the residues for each biphasic solvent system tested and the mixtures were vigorously vortexed. After standing for 30 minutes to allow phase separation, 1 mL aliquots of each phase were transferred to 1.5-mL Eppendorf tubes and evaporated to dryness under vacuum using a Genevac. Then, they were resolubilized in 400 µL of methanol/water 50:50 supplemented with formic acid 1% and analysed using UPLC-UV/Visible-MS. The partition coefficients K were calculated as follows by considering a CPC in descending mode.

$$K = A_U / A_L$$

Where A_U and A_L are the UPLC-UV peak areas of the compound considered in the upper and lower phases, respectively (measured at 280 nm for FA and DHC, and 320 nm for HCA).

To assess the relevance of using pH-ZRCPC for the fractionation of the different classes of polyphenolic compounds, the same procedure was applied adding an acid or base to the extract solubilized in the biphasic mixture. To determine the partition coefficient at a relatively elevated pH (noted K_{base}), sodium hydroxide was added and the pH measured in the aqueous phase. Two "elevated" pH were thus defined (about 5 and 8). Moreover, to determine the partition coefficient at acidic pH (noted K_{acid}), trifluoroacetic acid (TFA) was added to adjust the pH to 2 in the aqueous phase. K_{acid} was only determined for systems A and B.

2.3.2 Preparation of a two-phase solvent system for pH-ZRCPC

A biphasic solvent system consisting of ethyl acetate/*n*-butanol/water (3/2/5, v/v) was used. After vigorously shaking and leaving the mixture to thoroughly equilibrate in a separatory funnel until complete phase separation, the upper and lower phases were separated. Two retainer/eluter concentration ratios were tested. For the first pH-ZRCPC (CPC1), trifluoroacetic acid was added to the upper organic phase to obtain a final concentration of 2 mmol/L, and 50% sodium hydroxide was added to the lower aqueous phase to obtain a final concentration of 0.5 mmol/L (measured pH = 7.1). For the second pH-ZRCPC (CPC2), the TFA and NaOH concentrations were set at 10 mmol/L (measured pH = 7.36 for the lower phase).

2.3.3 Sample preparation from the crude apple extract

The solution for pH-ZRCPC was prepared as follows: 1 g of crude sample was dissolved in 5 mL of stationary phase (containing 5 µL TFA) and 5 mL of lower phase (without sodium hydroxide). The solution was filtered on a 0.45 µm PTFE membrane before fractionation using pH-ZRCPC.

2.3.4 Apparatus

pH-zone-refining CPC was carried out using an FCPC200® apparatus provided by Kromaton Technologies (Rousselet-Robatel, Annonay, France) equipped with a semi-preparative column of 200 mL (actual volume: 189 mL). The CPC system was coupled to a Gilson (Middleton, WI, USA) PLC 2020 preparative gradient pumping system comprising a binary high-pressure pump, a Rheodyne injection valve equipped with a 20 mL sample loop, a dual-wavelength UV detector, and a fraction collector.

2.3.5 pH-ZRCPC procedure

The pH-ZRCPC was carried out in descending mode (head-to-tail). The column was initially filled with the acidified organic stationary phase at a flow rate of 5 mL/min and a constant rotation speed of 1200 rpm. The crude apple extract solution was then injected and the fractionation was initiated by immediately pumping the aqueous mobile phase (sandwich injection mode). The eluter concentration depended on the CPC experiment considered. After an elution time of ten minutes, the elution solvent was continuously collected in a series of glass tubes (15 mL every 3 minutes). The final extrusion step of the column was achieved by pumping the organic phase after 250 min for CPC1 and 115 minutes for CPC2. Collection of the elution solvent was continued in the same way during the extrusion step.

The pH was determined manually using a portable pH meter (model 713, Metrohm, Hersiau, Switzerland) in aqueous CPC sample tubes only (from 16 to 259 minutes for CPC1 and 16 to 127 minutes for CPC2) because the pH values of the collected fractions became somewhat erroneous in the organic mobile phase during the extrusion step. When the pH exceeded 5, collected sample tubes were acidified to pH 3 to avoid autoxidation. Only CPC sample tubes of interest were analysed using UPLC-UV/Visible-MS to determine their phenolic composition according to the UV signals at 280 nm and 320 nm. When no absorbance was observed, the fractions were not analyzed. For the CPC, the choice of pooling or not the fractions collected was determined according to their detailed polyphenol composition obtained using UPLC-UV/Visible-MS analysis. After pooling, the fraction was concentrated under vacuum to remove any organic solvents. The aqueous concentrate was freeze-dried before the preparative reversed-phase chromatography step.

2.4 Preparative Reversed-Phase HPLC

The ultimate purification step was achieved using preparative scale reversed-phase HPLC with the same preparative HPLC gradient pumping system (PLC2020) as described in 2.3.4. For this fractionation, the pumping system was coupled to a preparative column (length, 220 mm; diameter, 47 mm) packed with Lichrospher 100 RP-18, 12 µm (Merck, Darmstadt, Germany).

The CPC2 pH-ZRCPC extract was solubilized in 5 mL acetonitrile/H_2O 20/80 acidified with formic acid 1% and filtered on a 0.45 µm PTFE membrane. The whole volume was injected onto the preparative column at ambient temperature with a flow rate set at 40 mL/min. The solvents comprised 0.1% (v/v) formic acid (A) and acetonitrile acidified with 0.1% (v/v) formic acid (B). The chromatographic conditions were as follows: 0-30 min, 20% B isocratic;

30-35 min, linear gradient to 90% B; 35-40 min, 90% B isocratic, then reconditioning column to 20% B. The UV signal was monitored at 280 nm and 320 nm. The elution fractions were collected manually and were analysed using UPLC-UV/Visible-MS.

The fractions containing only flavan-3-ols were pooled, evaporated under vacuum to remove any solvents, and freeze-dried. Finally, the purified flavan-3-ol extract was fully characterized as described above for the crude apple extract.

2.5 UPLC-UV/Visible-MS Analyses

All samples (crude, intermediate, and purified extracts) were analysed using the Acquity Ultra Performance LC System (Waters, Milford, MA) equipped with a degasser, a binary solvent manager, an autosampler, and a PDA detector used in the 190 nm - 500 nm range and connected to a Quattro Premier XE triple quadrupole mass spectrometer. The latter was equipped with an electrospray ionization source used in negative mode. Nitrogen was used as the nebulizer and desolvation gas. The source parameters were as follows: capillary voltage, 3 kV; cone voltage, 30 V; cone gas flow, 50 L/h; desolvation gas flow, 500 L/h; source temperature, 150°C; desolvation temperature, 250°C. Data were collected and processed using MassLynx software (V 4.0).

Samples (2 µL) were injected onto a UPLC reversed-phase column, Acquity UPLC BEH C_{18} (100 mm × 2.1 mm, 1.7 µm, Waters), with a flow rate of 0.35 mL/min and a column temperature set at 30 °C. The eluent was a gradient of 0.1% (v/v) formic acid (A) and acetonitrile acidified with 0.1% (v/v) formic acid (B). The elution gradient was applied as follows: initial, 3% B; 0−1 min, linear gradient to 7% B; 1−8 min, linear gradient to 13% B; 8-10 min, 13% B isocratic; 10-15.5 min, linear gradient to 20% B; 15.5−19 min, linear to 45% B followed by washing and reconditioning of the column. Under these chromatographic conditions, CQA and (+)-catechin are coeluted and were separated by applying the same elution gradient at a higher column temperature (45°C) thus allowing their quantification. Phenolic compounds were identified by LC-UV-MS analysis comparing the retention times, UV-Visible spectra, and molecular ions with those of available standards. Quantification was performed by integrating peaks on UV-visible chromatograms at 280 nm for flavan-3-ols and DHC, at 320 nm for HCA, and at 350 nm for FO. CAT, EC, PA_B2, CQA, PLZ and QCE-3-O-GAL were quantified according to their own calibration curves. Quantification of others compounds was carried out using a reference compound in the same phenolic class displaying a very similar UV-Visible spectrum. So, EC was used to quantify procyanidin trimer C1 (PA_C1), unknown procyanidin trimer (DP3) and procyanidin tetramer (DP4). Procyanidin

dimer B5 (PA_B5) was quantified using the response factor of PA_B2. FO were quantified using the one of QCE-3-O-GAL.

3 Results and Discussion

A crude polyphenol extract (TotPP) was prepared from a polyphenol-rich cider apple juice and its detailed polyphenol composition was determined. This extract was used as the starting material for optimizing preparative fractionation of apple polyphenols using pH-ZRCPC followed by reversed-phase preparative HPLC. First, several biphasic solvent systems, also including variations in TFA and NaOH concentrations, were tested on an analytical scale for the liquid/liquid fractionation of this extract. This allowed the partition coefficients of major phenolic compounds to be determined and the most suitable solvent system for pH-ZRCPC to be selected on a preparative scale. An intermediate extract no longer containing HCA was thus obtained and finally purified using Preparative HPLC producing a final extract named "MM-FA".

3.1 Detailed polyphenol composition of the crude apple extract (TotPP) obtained from a French cider apple juice purified on resin.

The TotPP extract comprised mainly three classes of phenolic compounds. As shown in table 2, HCA, which corresponds to the sum of CQA and PCQ (4-O-*p*-coumaroylquinic acid), accounted for more than one-third of the total polyphenol content (35.9%), followed by catechins and some procyanidin oligomers (26.5%), and dihydrochalcones (2.6%) (Table 2). The UPLC-UV/Visible-MS results obtained after the phloroglucinolysis reaction allowed the flavan-3-ol content to be assessed, which represented 463.3 g/kg of the dried crude extract with an average polymerization degree of 3.3. Therefore, from this crude polyphenol extract, an efficient fractionation procedure would allow the recovery without prior phloroglucinolysis of about 46% of flavan-3-ols, a part of procyanidins corresponding to oligomers from DP2 to DP4 (192.1 g/kg) and another part to the half of the procyanidins (198.2 g/kg) that were not quantified using direct reversed-phase UPLC analysis.

3.2 Optimization of a two-phase solvent system for pH-ZRCPC

For a solute distributed between two solvent phases, the partition coefficient K is usually expressed as the ratio of the amount in the stationary phase to that in the mobile phase, as in conventional liquid chromatography. The fractionation of two compounds using conventional CPC requires selecting a solvent system for which the ratio of partition coefficients K is greater than 1.5, with K values ideally ranging from 0.2 to 5.0. Thus, the K values of the main

compounds present in the crude polyphenol extract "TotPP" were first determined for a set of 11 solvent systems adapted from the Oka classification [58], in descending mode (mobile aqueous phase). With less polar solvent systems, all the polyphenol K values were lower than 1, suggesting that they were mainly partitioned in the aqueous phase (data not shown). Phenolic compounds are, therefore, generally fractionated with polar solvent systems [59].

Table 2. Composition of phenolic compounds in TotPP and MM-FA extracts (results expressed in g/kg of dry extract) and structural characterization of flavan-3-ols.

Phenolic compounds	TotPP	MM-FA extract
CQA	344	-
PCQ	14.9	-
EC	68.7	137.6
CAT	4.3	7.1
PA_B1	10.3	9.8
PA_B2	105.7	204.9
PA_B5	5.7	10.7
PA_C1	43.4	90.2
DP3	1.5	2.6
DP4	25.5	63.2
XPL	18.1	-
PLZ	8.0	-
Other procyanidins assayed after phloroglucinolysis	198.2	298.9
Total polyphenols purity (%)	84.8	82.5
Total flavan-3-ols purity (%)	46.3	82.5
Structural characterization of flavan-3-ols:		
(-)-epicatechin as PA extension units (%)	70	67.5
(-)-epicatechin as free or PA terminal units (%)	26.8	29.1
(+)-catechin as free or PA terminal units (%)	3.2	3.4
aDP	3.3	3.1

CQA: 5'-O-caffeoylquinic acid; PCQ: 4-O-*para*-coumaroylquinic acid; EC: (-)-epicatechin, CAT: (+)-catechin; PA_B1: procyanidin dimer B1; PA_B2: procyanidin dimer B2; PA_B5: procyanidin dimer B5; PA_C1: procyanidin trimer C1; DP3: unknown procyanidin trimer; DP4: procyanidin tetramer; XPL: phloretin 2'-O-xyloglucoside; PLZ: Phloridzin. PA: procyanidin. aDP: average degree of polymerization.

Table 3 shows the partition coefficients of each phenolic compound according to systems A to D. With system A, CQA and PCQ showed K values of 0.38 and 0.82, respectively. Therefore, it seems possible to isolate them from monomers (CAT, EC) and some of the procyanidin oligomers (PA_B5 and DP3) whose K values exceeded 1.7. However, the main procyanidin oligomers (PA_B2, PA_C1, DP4, and PA_B1) exhibited K values lower than 1, indicating that system A was not suitable for separating these compounds from HCA. Additionally, systems B to D were not selected in classic CPC mode as all the K values measured were too

high. Finally, regarding the strong disparity in K values, these solvent systems would not enable the separation of HCA without losing the catechin monomers and procyanidin oligomers.

K_{acid} and K_{base} were estimated to assess the applicability of the two-phase solvent systems. For successful separation using pH-ZRCPC, it is necessary to have $K_{base} << 1$ and $K_{acid} >> 1$, for an acidic analyte [36]. In other words, the molecule considered has to exhibit a polarity sufficiently different between its ionised and neutral forms to deeply modify its K values by modifying its acidity for a given solvent system. System A was discarded because K_{acid} was too low for all the compounds quantified in this system at pH 2 (Table 3). For system B, K_{acid} was much higher than 1 and K_{base} dropped significantly when the pH was adjusted to 7.9. At first glance, this solvent system was suitable for pH-ZRCPC. However, we noticed the appearance of an orange colour after adding sodium hydroxide to the crude apple extract, suggesting autoxidation of some of the phenolic compounds in those alkaline conditions. This was confirmed using UPLC-UV/Visible-MS analysis by comparing the amounts of phenolic compounds in the solvent systems before and after alkalization. When the aqueous phase was basic (measured pH = 7.93), degradation of procyanidins, and (-)-epicatechin (EC) occurred (Table 4). We also noticed an increase in catechin (CAT) content, which is likely caused by the epimerisation of (-)-epicatechin into (-)-catechin due to the alkaline conditions [60].

To avoid the autoxidation of phenolic compounds, we decided to reduce the pH of the aqueous phase to 5.2. As the pKa of CQA and PCQ are 2.6 and 3.4, respectively, these molecules are almost totally ionised at pH 5.2, making pH-ZRCPC possible. For this pH (5.2), neither a colour change nor a decrease in polyphenol content was observed, showing a satisfactory recovery of polyphenols (4). The new K_{base} values at pH 5.2 for CQA and PCQ were suitable for solvent system B, although they were not largely below 1 (0.42 and 0.51, respectively). K_{base} values were very similar for solvent systems C and D (Table 3). Fortunately, the distribution of procyanidins in the two phases did not change extensively and the K_{base} was still higher than 1, making pH-ZRCPC possible with these three systems. Finally, it was not necessary to determine K_{acid} with the solvent systems C and D for a practical reason: at pH 5.2, after shaking, the phases of these two systems were not totally separated suggesting they were not suitable for CPC fractionation. Finally, among the solvent systems tested, system B, with a pH shift from 2.15 to 5.2, was the most appropriate for our purpose.

371 Table 3. Partition coefficients of phenolic compounds in TotPP extract according to different solvent systems: Ethyl acetate/*n*-butanol/water (v/v)
372 with A (5/0/5), B (3/2/5), C (2/3/5), and D (1/4/5).

Solvent system	pH	Partition Coefficient	Phenolic compounds											
			CQA	PCQ	EC	CAT	PA_B1	PA_B2	PA_B5	PA_C1	DP3	DP4	XPL	PLZ
A	2.06	Kacid	0.89	1.69	2.64	1.85	0.24	0.57	2.38	0.39	1.98	0.19	0.23	5.87
A	4.02	K	0.38	0.82	2.51	2.13	0.30	0.60	2.58	0.35	1.78	0.15	0.23	6.55
B	2.15	Kacid	7.34	14.71	24.51	13.29	4.24	14.90	34.93	16.39	-	15.20	8.47	47.11
B	4.27	K	3.23	4.16	12.71	14.2	2.79	8.31	-	13.36	-	16.21	8.29	54.81
B	5.20	Kbase	0.42	0.51	12.27	11.07	3.77	8.38	37.09	13.19	-	16.41	8.14	52.43
B	7.93	Kbase	0.00	0.01	0.25	0.07	0.05	0.07	0.14	0.04	-	0.03	0.04	0.13
C	4.33	K	2.95	4.11	10.58	12.05	2.42	6.94	36.61	11.08	-	12.64	10.47	55.76
C	5.25	Kbase	0.44	0.55	10.19	8.35	3.43	6.92	24.57	10.63	-	14.29	9.98	49.51
D	4.27	K	2.87	4.10	7.22	10.11	1.41	4.08	20.61	5.69	-	5.24	9.35	40.65
D	5.23	Kbase	0.42	0.56	7.04	6.83	1.98	4.24	14.72	5.99	-	6.05	9.23	49.47

373 CQA: 5'-O-caffeoylquinic acid; PCQ: 4-O-*para*-coumaroylquinic acid; EC: (-)-epicatechin, CAT: (+)-catechin; PA_B1: procyanidin dimer B1;
374 PA_B2: procyanidin dimer B2; PA_B5:procyanidin dimer B5; PA_C1: procyanidin trimer C1; DP3: unknown procyanidin trimer; DP4:
375 procyanidin tetramer; XPL: phloretin 2'-O-xyloglucoside; PLZ: Phloridzin.

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Table 4. Remaining phenolic compounds (%) in the two alkalized solvent system B compared to system B without adding a base.

Compound	pH 7.9	pH 5.2
CQA	59	88
PCQ	55	100
EC	60	100
CAT	184	89
PA_B1	80	126
PA_B2	64	104
PA_B5	33	83
PA_C1	67	104
DP3	0	86
DP4	66	108
XPL	81	100
PLZ	86	101

CQA: 5'-O-caffeoylquinic acid; PCQ: 4-O-*para*-coumaroylquinic acid; EC: (-)-epicatechin, CAT: (+)-catechin; PA_B1: procyanidin dimer B1; PA_B2: procyanidin dimer B2; PA_B5: procyanidin dimer B5; PA_C1: procyanidin trimer C1; DP3: unknown procyanidin trimer; DP4: procyanidin tetramer; XPL: phloretin 2'-O-xyloglucoside; PLZ: Phloridzin.

3.3 First pH-ZRCPC fractionation (CPC1)

One gram of crude apple extract was fractionated using pH-ZRCPC with the biphasic solvent system B selected above composed of ethyl acetate/*n*-butanol/water (3/2/5, v/v). Reverse displacement mode was used by choosing the lower aqueous phase as the mobile phase (descending mode or head-to-tail). The TotPP extract was solubilized at acidic pH to prevent autoxidation of the phenolic compounds. In addition, to keep the pH below 5.2 during elution, a first fractionation was conducted with a low concentration of NaOH (0.5 mM) in the aqueous mobile phase. We deliberately chose a higher retainer concentration to ensure that the phenolic compounds would be in their protonated form. Figure 1a shows the chromatogram of CPC1 with 2.3 mM of TFA in the organic stationary phase and 0.5 mM of sodium hydroxide in the aqueous mobile phase. A large peak was eluted between 100 and 190 minutes and was attributed to HCA due to the intense UV signal observed at 320 nm and the UPLC-MS analyses of the CPC fractions from **a** to **g** (supplementary data). However, the pH curve was not consistent with what was expected for pH-ZRCPC. The pH curve should be flat throughout the HCA elution time and the characteristic rectangular shape of the peak was not

observed. In addition, CPC sample tubes analyses showed that the main procyanidin (PA_B2) eluted at 172 min in fraction **f** and coeluted with HCA. This confirmed that these conditions were not suitable for the efficient separation of hydroxycinnamic acids derivatives from procyanidin oligomers. These results can be interpreted with regards to the study of Ito *et al.*, as the eluter plays the role of a counterion for the target compound the molar concentration of the eluter largely determines the molar concentration of each target compound in the fraction. Therefore, a higher eluter concentration yields a higher concentration of each target compound in a shorter elution time. However, if the concentration of the target compound exceeds its solubility in the stationary phase, the target compound will precipitate in the column and plug the channel, thereby damaging the column [36]. In our case, the concentration of OH^- ions, which played the role of counterions for the target compounds, was not high enough based on the theoretical amounts of HCA to be fractionated (360 mg).

3.4 Second pH-ZRCPC fractionation (CPC2)

Considering the parameters that were likely responsible for the failure of CPC1 fractionation, another pH-ZRCPC, named CPC2, was carried out with the same quantity of sample injected and increasing the retainer and eluter concentrations to 10 mM for each phase of the biphasic solvent system. Indeed, Ito *et al.* showed that these equimolar concentrations of retainer and eluter (10 mM) produce satisfactory separation in most cases [36]. The retainer concentration was high enough to ensure the protonation of all ionizable phenolic compounds and therefore to promote their trapping in the organic stationary phase. In addition, the eluter in the aqueous mobile phase allowed the progressive neutralization of the retainer (H^+) and the displacement of the ionisable compounds from the stationary phase to the aqueous mobile phase.

In comparison with CPC1, increasing the eluter concentration considerably shortened the retention time (Figure 1b). After the solvent front of the mobile phase (sharp peak at 37 min), two zones could be clearly distinguished. UV chromatograms at 280 nm and 320 nm showed an almost rectangular peak eluted from 49 to 100 minutes with a stable pH value at 4.4. We also noticed a small sharp shoulder at the end of the large rectangular peak (retention time at 97 min). The UPLC-MS analyses revealed that the rectangular peak corresponded to CQA (fractions 2 to 8), and the sharp shoulder corresponded to PCQ (supplementary data). The UV signal at 320 nm was in accordance with the presence of HCA, whose maximum absorbance wavelength is about 320 nm. In these conditions, fractions containing CQA and PCQ overlapped.

After 100 min, as the pH exceeded 5, the fractions were immediately acidified to avoid autoxidation of phenolic compounds. Then, elution-extrusion was started manually at 115 min. This elution-extrusion mode, which consists in replacing the aqueous mobile phase with the organic phase, has already been used for pH-ZRCPC to elute more hydrophobic compounds and reduce the elution time [61]. The second zone, from 128 min to 164 min, was a set of three intense co-eluting peaks showing high absorbance at 280 nm and only weak absorbance at 320 nm, suggesting that they were mainly flavan-3-ols. Indeed, UPLC-MS analyses revealed that the first one was composed of procyanidin dimer PA_B2 with XPL (dihydrochalcone), which also explained the weak signal at 320 nm (fractions 12 and 13). The second one comprised (-)-epicatechin, procyanidin trimer PA_C1, and procyanidin tetramer DP4 (fraction 14). The third one was composed of PLZ, PA_B5, CAT, and DP3 (Fractions 15 to 18). For these fractions, the signal at 320 nm showed two co-eluting peaks due to the existence of PLZ and several quercetin glycosides (whose λmax is at 350 nm). The latter, present in very low quantities in the TotPP extract (and not quantified), were clearly detected by UPLC-UV/Visible-MS due to the enrichment by pH-ZRCPC. Lastly, the UPLC-MS analyses of fractions 9 and 10 highlighted that the procyanidin dimer PA_B1 was eluted before extrusion, after the elution of HCA. These results suggest that the solvent system should be improved to also recover this procyanidin dimer.

Finally, the CPC fractions between 109 min and 166 min were pooled to obtain 532 mg of extract. This quantity was above the expected amount (489 mg). This difference could be explained by the formation of salts (sodium trifluoroacetate). We consider that we succeeded in obtaining an intermediary extract containing the flavan-3-ols representative of the initial extract, with dihydrochalcones and flavonols.

3.5 Ultimate purification using Preparative HPLC

As XPL, PLZ, and flavonols are less polar compounds than procyanidin oligomers, they can be easily separated from procyanidins using Reversed-phase HPLC with a C18 column. Salts produced during pH-ZRCPC are also removed by being eluted at the beginning of the reversed-phase HPLC. The pH-ZRCPC extract was solubilized in 20% acetonitrile. With isocratic chromatographic conditions at 20% acetonitrile as well, all procyanidin oligomers were eluted earlier than the other phenolic compounds. The UV 280 nm chromatographic profile was divided into 3 zones (Figure 2). The analyses of fractions showed that procyanidin oligomers and catechin monomers were eluted in the first one ranging from 6.5 min to 22.2 min (see supplementary data), only 0.15% of unwanted compounds were present in those

fractions. Consequently, they were pooled to obtain the final extract, named MM-FA extract. The second one from 22.2 min to 33 min corresponded to dihydrochalcones and flavonols. The third zone was not recovered as it corresponded to the washing of the column.

3.6 Composition and structural characterization of MM-FA extract

The MM-FA extract (409 mg) was purified from 1 g of the initial crude extract. Overlaying the TotPP and MM-FA chromatograms (Figure 3) showed that our strategy enabled the enrichment of flavan-3-ols without HCA (CQA and PCQ) and dihydrochalcones (XPL and PLZ). UPLC-UV/Visible-MS analysis confirmed that none of them were present in the final purified extract. The final tannic powder was characterized and thus can be considered as a pure apple flavan-3-ol extract. The catechin monomers (EC and CAT) and individually quantifiable procyanidin oligomers (from DP2 to DP4) represented 14.5 % and 38.1% of the purified extract (MM-FA), respectively (Table 2). Procyanidin dimer B2 alone accounted for 20.5 % of the fraction. Other minor peaks were also observed. They corresponded to other procyanidin oligomers from DP3 to DP6. HPLC-UV/Visible-MS analysis after phloroglucinolysis allowed the total flavan-3-ols content to be determined, which represented 825 g/kg. Therefore, others procyanidin oligomers and polymers not separated by direct analysis using UPLC represented circa 30% of the extract. Noticeably, as previously mentioned for freeze-dried procyanidin extracts [62], a significant quantity of bound water (estimated at 2-3 water molecules per catechin unit constituting the procyanidin structures) likely remained present in the purified extract after freeze-drying [63]. This probably explains the 82.5% of total polyphenols in the final MM-FA extract. Complementary information was provided regarding the nature and proportions of the flavan-3-ols units entering in the composition of the MM-FA extract (Table2). Indeed, extension units were exclusively (-)-epicatechin accounting for 67.5% of total FA units. Terminal procyanidin units or free catechin units were essentially (-)-epicatechin (29%), (+)-catechin accounting only for 3%. Lastly, the recovering of flavan-3-ols, corresponding to the ratio of the quantity of flavan-3-ols in the final extract (337 mg) on the one in 1 g of crude extract (463 mg) was 73%. This percentage of recovery is satisfactory, considering that a part of PAB1 was not totally recovered, and that there were unavoidably losses during the numerous steps of this methodology (fraction analysis, remaining in the syringe during injection, highly polymerized procyanidins more hydrophobic and eluting later during preparative RP-HPLC). Despite this, the average polymerization degree (aDP) of the flavan-3-ols determined in the MM-FA extract (3.1) was very close to that of the initial TotPP extract (3.3) (Table 2).

4 Conclusion

CPC combined with a pH-displacement mode and followed by preparative reversed-phase chromatography is an efficient methodology for the quantitative purification of flavan-3-ol monomers and oligomers. The method was particularly relevant to properly discard on a preparative scale, hydroxycinnamic acid derivatives, dihydrochalcones and flavonols from a crude apple polyphenol extract. In this study, a highly purified flavan-3-ol fraction (409 mg) was obtained. Purification yield was 73% and the purity, estimated to 83%, is likely underestimated considering the presence of hydration water. The quantitative analyses of catechin monomers and procyanidin oligomers, as well as their structural characterization (free, terminal and extension FA units) showed that the end fraction was clearly representative of the initial composition of the crude apple extract. The pure extract will prove to be of great use to investigate both organoleptic and nutritional properties of flavan-3-ols monomers and oligomers present in apple and apple-derived beverages.

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6. Figure captions

Fig.1. pH-ZRCCC chromatograms of the crude sample from 1 g TotPP extract. solvent system used: ethyl acetate/*n*-butanol/water (3/2/5, v/v); flow rate: 5 mL/min; revolution: 1200 rpm. a: 2.3 mM of TFA in the stationary phase and 0.5 mM NaOH in the mobile phase. b: retainer and eluter at 10 mM in the stationary and mobile phases, respectively. The graphics with thick and thin lines correspond to the UV signals at 280 and 320 nm, respectively (primary axis).

Fig.2. Preparative reversed-phase HPLC chromatogram of pH-ZRCPC extract (532 mg). Bold line: UV signal at 280 nm; thin line: UV signal at 320 nm (primary axis). The dotted line corresponds to the acetonitrile gradient (%).

Fig.3. Comparison of analytical chromatographic profiles at 280 nm of the TotPP extract (dotted line) and the MM-FA extract after pH-ZRCPC followed by preparative HPLC (full line). UV signal was normalized regarding the more intense peak of each chromatogram.

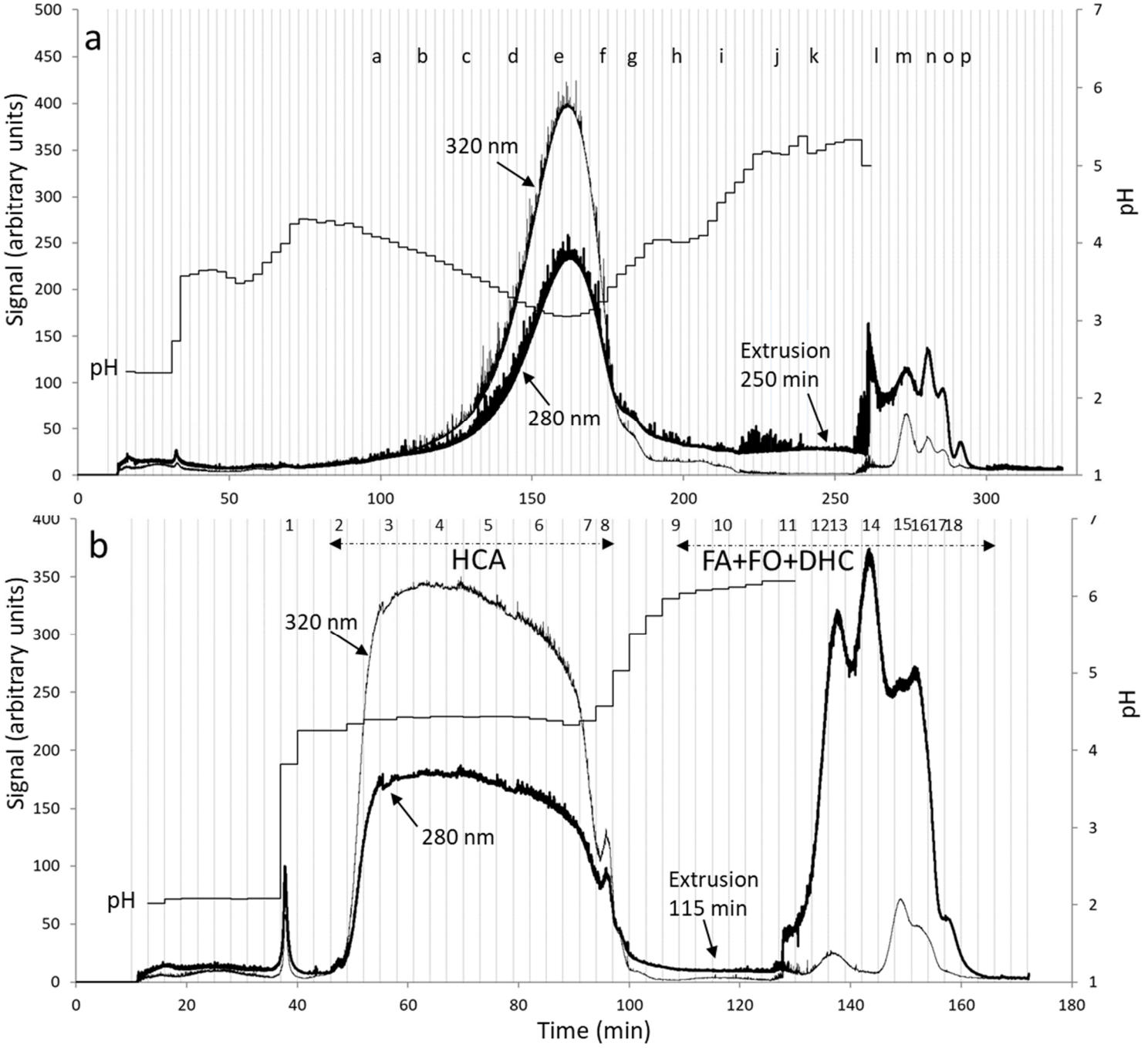
a
a b c d e f g h i j k l m n o p
320 nm
280 nm
pH
Extrusion
250 min
Signal (arbitrary units)
pH
b
1 2 3 4 5 6 7 8
HCA
9 10 11 12 13 14 15 16 17 18
FA+FO+DHC
320 nm
280 nm
pH
Extrusion
115 min
Signal (arbitrary units)
pH
Time (min)

Figure 1

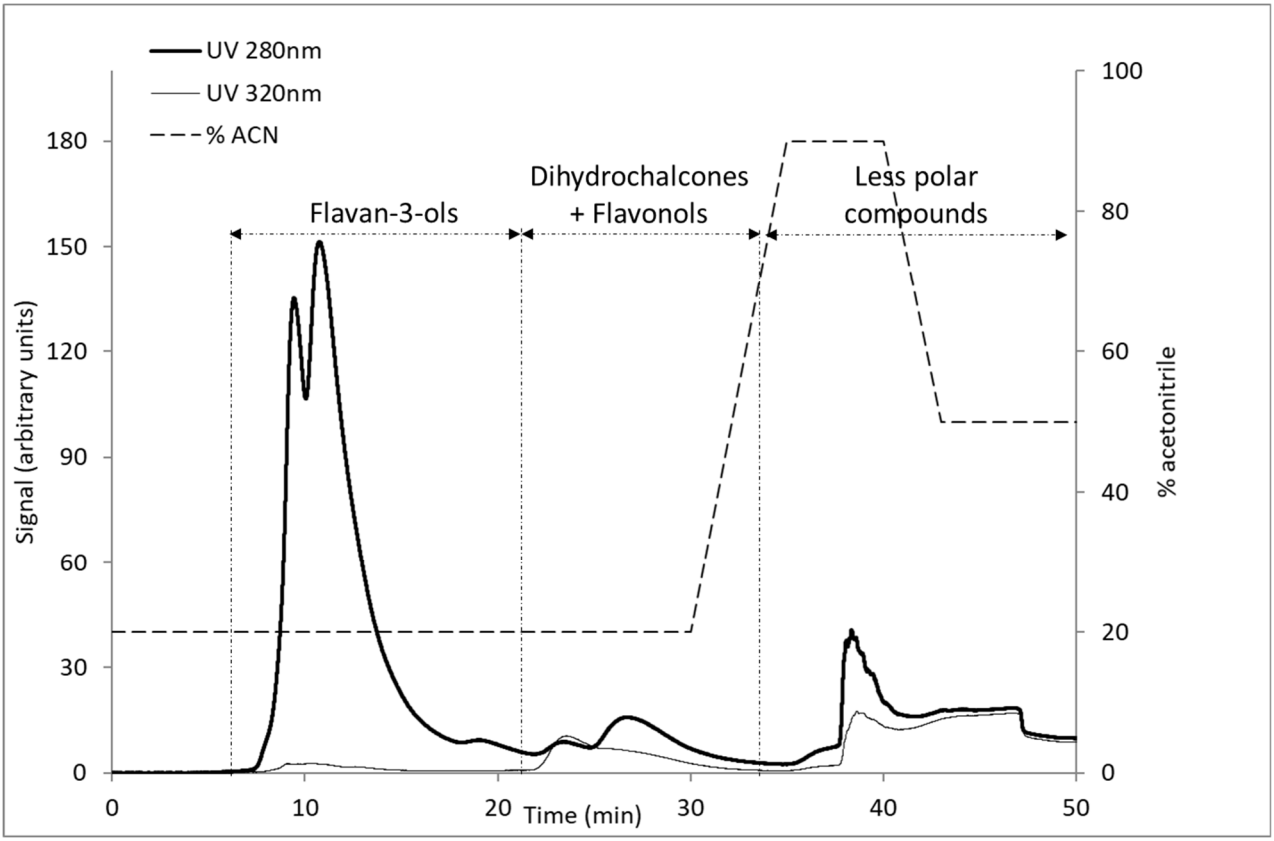
UV 280nm
UV 320nm
% ACN
Flavan-3-ols
Dihydrochalcones
+ Flavonols
Less polar
compounds
Signal (arbitrary units)
% acetonitrile
Time (min)
0
30
60
90
120
150
180
0
20
40
60
80
100
0
10
20
30
40
50

Figure 2

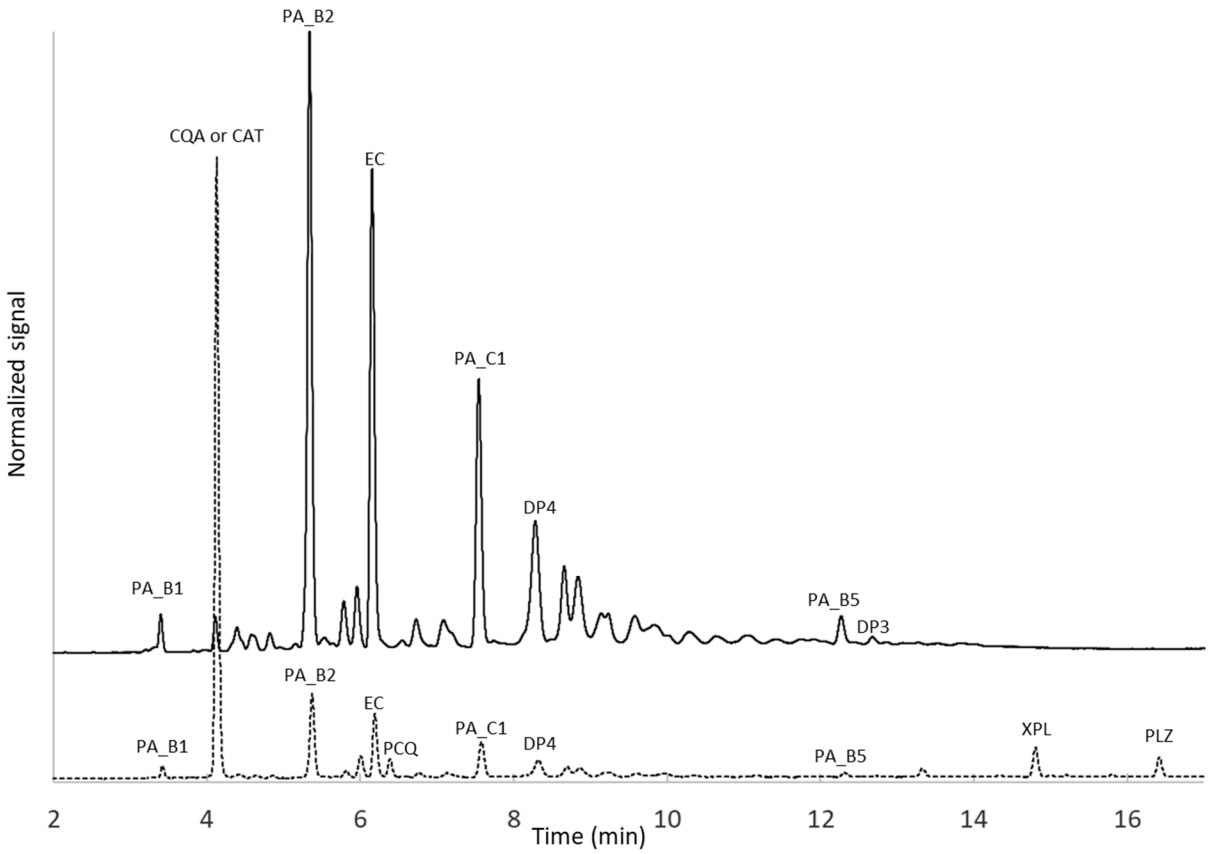
PA_B2
CQA or CAT
EC
PA_C1
DP4
PA_B1
PA_B5
DP3
Normalized signal
PA_B2
EC
PA_B1
PCQ
PA_C1
DP4
PA_B5
XPL
PLZ
2
4
6
8
10
12
14
16
Time (min)

Figure 3